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13. ABSTRACT (Maximum 200)			The goal of this project was to develop short peptides derived from the sequence of the human estrogen receptor that might serve as a novel class of receptor antagonists. By interacting with the dimerization motif of this protein, these peptides were intended to interfere with subunit dimerization and therefore to disrupt the DNA-binding activity of this key regulatory factor. Peptides were purchased and screened for their ability to interfere with the DNA-binding ability of this receptor using an <i>in vitro</i> gel mobility shift assay. Five peptides were identified that specifically and reproducibly disrupted DNA-binding. Their inhibition was shown to be concentration dependent. These results were complemented by chemical mutagenesis experiments that identified two residues falling within regions defined by the inhibitory peptides (Ala ⁵⁰⁵ and Leu ⁵¹¹) whose mutation also interfered with DNA binding. These data are discussed in the context of recent information detailing the X-Ray crystal structure of the hormone-binding domain of the estrogen receptor. Experiments were also undertaken to test the transcriptional inhibitory activity of these active peptides in a transient transfection system. This ultimately proved unsuccessful due to difficulties in achieving efficient peptide uptake by cultured cells. The promising preliminary results from this work supported the filing of an invention disclosure that is currently under consideration for further development.		
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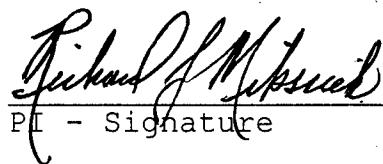
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INTRODUCTION

Title: Development of Novel Peptide Inhibitors of the Estrogen Receptor

PI: Richard J. Miksicek

Summary of Administrative Matters

This USAMRDC Breast Cancer Research IDEA Award was made to Richard J. Miksicek, who currently holds a primary appointment as Assistant Professor of Physiology at Michigan State University (MSU). This report summarizes the third and final year of this award, through 10/30/97. During the first year of this award, the P.I. (Dr. Miksicek) accepted a new position (effective 07/01/95) in the Department of Physiology at MSU, retaining an appointment as Adjunct Research Assistant Professor in the Department of Pharmacological Sciences of SUNY @ Stony Brook. At that time, permission was requested from the US Army MRMC through the awardee institution (The Research Foundation of SUNY) to effectively change the site of performance of this project from SUNY @ Stony Brook to MSU by establishing a research subcontract between these institutions. This research subcontract stipulated that Dr. Sandra Haslam (Professor of Physiology and Director of the MSU Breast Cancer Program) be named as Principal Investigator for the MSU subgrant with Dr. Miksicek continuing to serve as P.I. on the primary award to SUNY @ Stony Brook. In addition, Dr. Miksicek acted as Principal Co-Investigator at MSU with primary responsibility for the conduct of research. This subcontract was accepted by both institutions on 03/28/96 to become effective retroactively to 07/01/95. Progress on this project was substantially delayed between 07/01/95 and 03/28/96 pending negotiation of the research subcontract and release of research funds at MSU to the PI and Co-PI for the payment of salaries, the purchase of research supplies, and the costs of services. As a consequence of this delay, a no-cost extension was requested from the US Army MRMC to extend the period of this project through 10/30/97, without additional funds. This extension was granted to the Research Foundation of SUNY and to its primary subcontractor, MSU, enabling work to continue towards completion of the original specific aims of this project. The following represents the final technical report summarizing significant accomplishments for the entire award period (10/01/94 through 10/30/97).

Scope of the Project

The aim of this project was to explore a novel mechanism for disrupting estrogen receptor (ER) function, with the hope of developing a new class of estrogen antagonists. The general aim was to investigate the potential of disrupting subunit dimerization in order to block the transcriptional stimulatory activity of ER. Essentially all inhibitors of steroid receptors that have been developed to date represent steroid analogues that act as hormonal antagonists by binding to the ligand-binding site of these proteins. Rather than stimulating receptor activity as do the physiologically important steroids, these "antihormones" block receptor function, presumably by failing to induce a transcriptionally active conformation in the receptor protein. As an alternative to hormonal antagonists, we tested the possibility of using peptides to disrupt ER function. These peptides were designed to mimic the dimerization interface (or other important surfaces of the ER protein) and to interfere with subunit association, thereby preventing efficient DNA-binding by receptor. Results summarized below demonstrate that this strategy represents a feasible mechanism to

interfere with DNA-binding by ER *in vitro*. If this approach can also be proven to function *in vivo*, it would have several important advantages over classical hormonal antagonists. Peptide inhibitors (or small molecules designed to mimic their 3-dimensional structure) should be relatively insensitive to competition by endogenous estrogens such as estradiol. They should also remain effective in tumors that have acquired resistance to currently available estrogen antagonists, and they would likely display very different pharmacokinetic and pharmacodynamic properties compared with hormonal antagonists.

The original statement of work for this project included the following specific tasks:

Task 1, to identify the minimal region of the ER polypeptide able to inhibit the DNA-binding and transcriptional activity of intact ER when co-expressed in cultured cells (months 1-18)

- a. to develop a series of plasmids for expressing progressively smaller portions of the ER open reading frame (months 1-6)
- b. to perform gel mobility shift assays to ascertain which fragments of ER block DNA-binding when expressed together with intact ER (months 6-12)
- c. to confirm that fragments of the ER open reading frame that block DNA-binding activity also function as dominant inhibitors of ER function *in vivo* (months 12-24)

Task 2, to identify short peptides modeled after the ER dimerization motif that act as inhibitors of DNA-binding and subunit association (months 1-24)

- a. to obtain synthetic ER peptides (months 1-3)
- b. to test the effect of synthetic peptides on the DNA-binding activity of ER using an *in vitro* gel mobility shift assay (months 3-12)
- c. to characterize the inhibitory potency of active peptides and attempt to optimize the effect by testing additional overlapping peptides (months 6-24)
- d. to perform feasibility experiments based on liposome-mediated peptide transfer to assess the ability of selected peptides to inhibit ER function in cultured cells (months 12-24)

EXPERIMENTAL METHODS

Plasmids and transfection methods.

The plasmids used for the transfection experiments described below included pERE-TK-CAT, an estrogen responsive chloramphenicol acetylase reporter plasmid (1) and a series of cytomegalovirus (CMV) promoter-driven ER expression plasmids, constructed in either pCMV4 (2), pcDNA3, or pCR3.1 (InVitrogen Corp., San Diego, CA). Regions of the ER polypeptide that are expressed by the plasmids used in these studies are depicted in figure 1. These plasmids, as well as methods used for transient transfection by calcium phosphate co-precipitation were described in greater detail in previous progress reports. The inhibitory potential of expressed fragments of ER were assessed by measuring the activity of chloramphenicol acetyltransferase (CAT) enzyme produced in cells co-transfected with the reporter plasmid and intact receptor alone (pCMV-ER), or together with the various ER mutants depicted in figure 1. Levels of ER expression were confirmed to be comparable by western blot analysis using an ER-specific monoclonal antibody (Mab-17) previously developed in this laboratory (3).

Custom synthesis of estrogen receptor peptides.

A series of 40 synthetic ER peptides were obtained by custom synthesis from Chyron Corp., La Jolla, CA). Their sequences are provided in Appendix A. The positions of these peptides within the reading frame of the ER protein are depicted in figure 2. Peptides # 24, 5, 42, 6, 10, and 14 overlap with the peptides proposed in the original grant application; they were redesigned before being synthesized. As described in a previous progress report, advances in the efficiency and economy of solid phase peptide synthesis enabled us to have this more extensive series of peptides synthesized at a reasonable cost. To mimic the natural electrostatic charges of each peptide, the amino-terminal (P_{37}) and carboxy terminal (P_{31}) peptides were synthesized with free amino and carboxyl groups, respectively. All of the remaining peptides were N-terminally acetylated or C-terminally amidated. Due to differences in size, charge, amino acid content, and hydrophobicity, these peptides display a wide range of apparent solubilities. The peptides were dissolved in 10% acetic acid and were used without further purification. Each of the 40 synthetic ER peptides was screened at various dilutions for the ability to interfere with DNA-binding using a standard gel mobility shift assay involving a consensus (palindromic) ERE.

Gel mobility shift assay for measuring interference of DNA binding by synthetic ER peptides.

Measuring the DNA-binding activity of ER and its inhibition by synthetic peptides was performed as detailed in an earlier progress report. Briefly, whole cell extracts prepared from Cos-7 cells that were transfected with pCMV-ER (a highly efficient ER expression plasmid) and treated for two hours with 10^{-8} M 17β -estradiol (E_2), prior to harvesting and preparing a 0.4 M KCl whole cell extract. Conditions used for our gel mobility shift assays have been previously described (3). To document the specificity of the reactions, control DNA-binding reactions were performed with equal amounts of extract (lacking ER) from pCMV4 vector-transfected Cos-7 cells. In addition, control DNA binding reactions containing 1 μ l of an ER-specific monoclonal antibody (3) were included to confirm the assignment of the ER/DNA complex.

Mutagenesis of the estrogen receptor.

Amino acid substitution mutants within selected portions of the ligand binding domain of ER were generated by random chemical mutagenesis using sodium bisulfite (4). Mutations were targeted to suspected dimerization motifs using a gapped-duplex approach. Point mutants used in this study included: T371I, L372F, H373Y, L378F, C381S, A382V, A505V, L509F, L511F,

S512L, and H516Y. (The nomenclature used for designating amino acid substitution mutants involves the single letter code for the natural amino acid, the residue within the ER polypeptide, and the single letter code for the mutated amino acid.)

Transfection conditions for assessing inhibitory activity of synthetic ER peptides.

In an attempt to assess the potential of synthetic ER peptides to interfere with activation of gene expression *in vivo*, assays were attempted using electroporation with an ECM600 electrocell manipulator to simultaneously introduce peptides and transfection plasmids into HeLa cells. A variety of electroporation conditions were tested, with voltages ranging from 125-500 v and capacitance settings varying between 1,200 to 3,000 microFarads. Peptide concentrations in the electroporation reaction were estimated to be approximately 0.04-0.2 microMolar. The goal of this approach was to attempt to simultaneously introduce selected synthetic ER peptides into HeLa cells along with an ER expression plasmid (pCMV-ER) and an estrogen-responsive reporter plasmid (pERE-TK-CAT) in order to look for inhibition of an ER mediated transcriptional response.

RESULTS

Delineation of portions of the ER polypeptide with inhibitory potential.

The ER protein consists of 595 amino acids that are assembled from eight protein coding exons of its gene. It is well established that this full-length receptor functions as an effective positive regulator of gene transcription when tested on its cognate hormone response element, the palindromic ERE (5'-AGGTCA_nnTGACCT-3'). Activation of transcription in response to E₂ requires not only that the DNA- and hormone-binding domains of the receptor are intact, but also requires that this protein can interact with itself to form homologous dimers as well as with other transcription factors and receptor coactivators to assemble into an active transcription complex. Favorable protein-protein interactions that are mediated by active surfaces on solvent-exposed faces of the correctly folded protein are thus critical for receptor function. This predicts that peptides and small molecules that are able to bind to these active surfaces may be able to interfere with dimerization, DNA-binding, or transcriptional activation by ER.

To investigate whether this represents a useful approach for the empirical design of novel inhibitors of ER function, a series of strategies were taken to identify "functionally critical surfaces" of the ER protein. The first approach involved use of deletion mutagenesis to attempt to delineate minimal regions of the ER peptide that display dominant negative activity. This approach makes use of the well established modularity of nuclear receptors and exploits the observation that destruction of one or more functional domain within the protein (while leaving other domains intact) can in some cases result in proteins that not only fail to activate transcription, but that actually inhibit the function of co-expressed intact receptor in a genetically dominant fashion. This inhibition presumably involves the ability of the mutated receptor to recruit intact receptors or crucial ancillary transcription factors into non-productive complexes. Figure 1 shows a summary of the behavior of seven ER deletion mutants that were tested for dominant negative activity in receptor co-transfection assays. Two independent endpoints (5) were used for these experiments: a) the ability of ER mutants to interfere with DNA-binding by intact ER as measured using an *in vitro* gel mobility shift assay and b) the ability of the mutants to block stimulation of gene expression by intact ER in a reporter gene co-transfection assay. As seen in figure 1, two of the seven mutants tested (ERΔE3 and ERΔE5) blocked transcriptional activation, but only one of these (ERΔE3) apparently did so (at least in part) by interfering with DNA binding. Since the two inhibitory mutants failed to share any common domains that were lacking among the inactive mutants, these results do not allow the simple assignment of a critical domain within ER that, when expressed in isolation, is able to block receptor function. Rather, they appear to indicate that

multiple mechanisms are likely to exist for dominant inhibition of ER function and that the disposition (i.e., protein context) or conformation of inhibitory domains is critical. These co-transfection experiments therefore represent a "proof of principle" that subfragments of the ER peptide exist that can exert an inhibitory effect on intact ER, but they do not go beyond the already accepted finding of ourselves (5) and others (6) that dominant negative mutants of ER exist. Due presumably to problems involved in correct folding of the ER deletion mutants analyzed, this approach was not effective in identifying "minimal inhibitory regions" of the ER polypeptide. Accordingly, this approach was not further pursued.

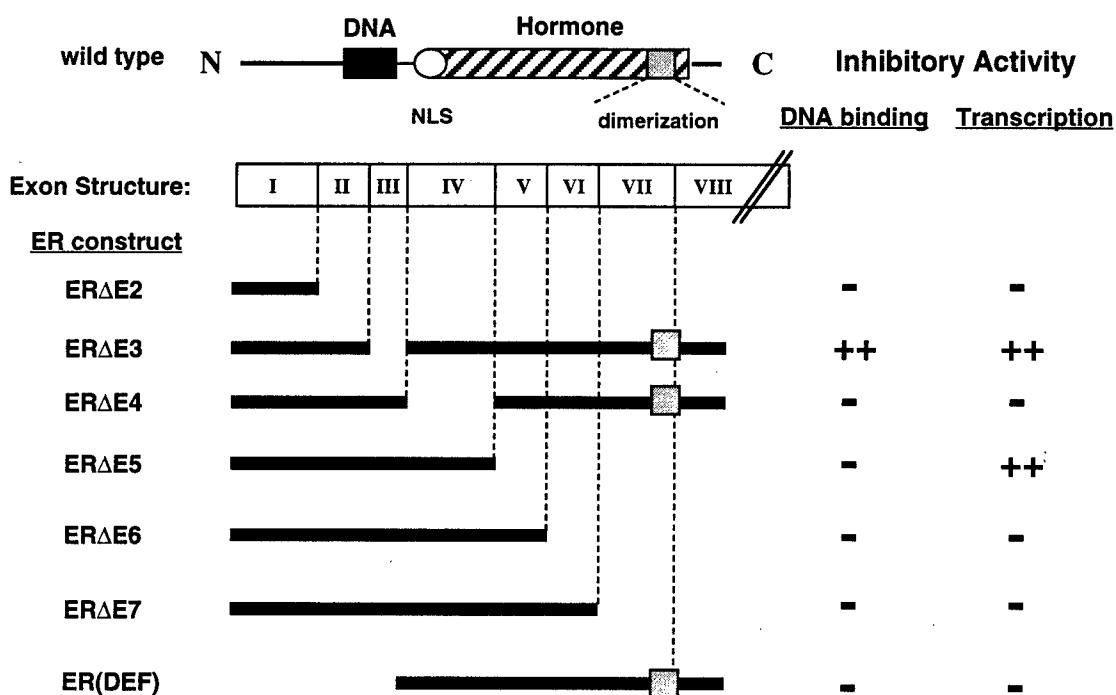


Fig. 1. Inhibitory effects of ER deletion mutants on the DNA-binding and transcriptional activities of wt ER

Screening synthetic ER peptides for interference with DNA-binding by intact receptor.

Considering the limited value of the co-transfection experiments described above, our efforts focused on a second strategy to identify "functionally critical surfaces" of the ER peptide. This approach was based on the use *in vitro* gel mobility shift assays to determine if synthetic peptides comprising sequences of the ER polypeptide (see figure 2) were able to interfere with the DNA-binding activity of ER. Each assay included control DNA-binding reactions performed with the [³²P]-ERE probe alone, with extracts from mock-transfected Cos-7 cells, and with extracts from Cos-7 cells that had been transfected with the ER expression vector pCMV-ER. DNA-binding reactions containing approximately 20 pmol of a single synthetic ER peptide together with a constant amount of Cos-7 / ER extract were then compared with a control reaction lacking added peptide. Representative gels from a series of experiments that systematically screened through each of the 40 synthetic peptides are presented in figure 3.

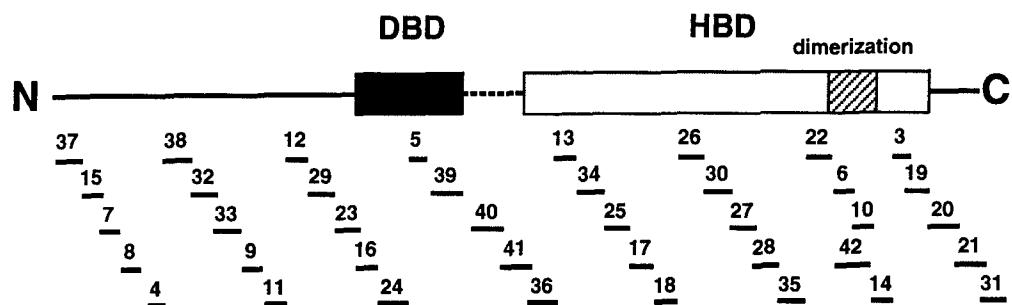


Fig. 2. Synthetic ER peptides used in this study and their locations within the reading frame of the ER protein

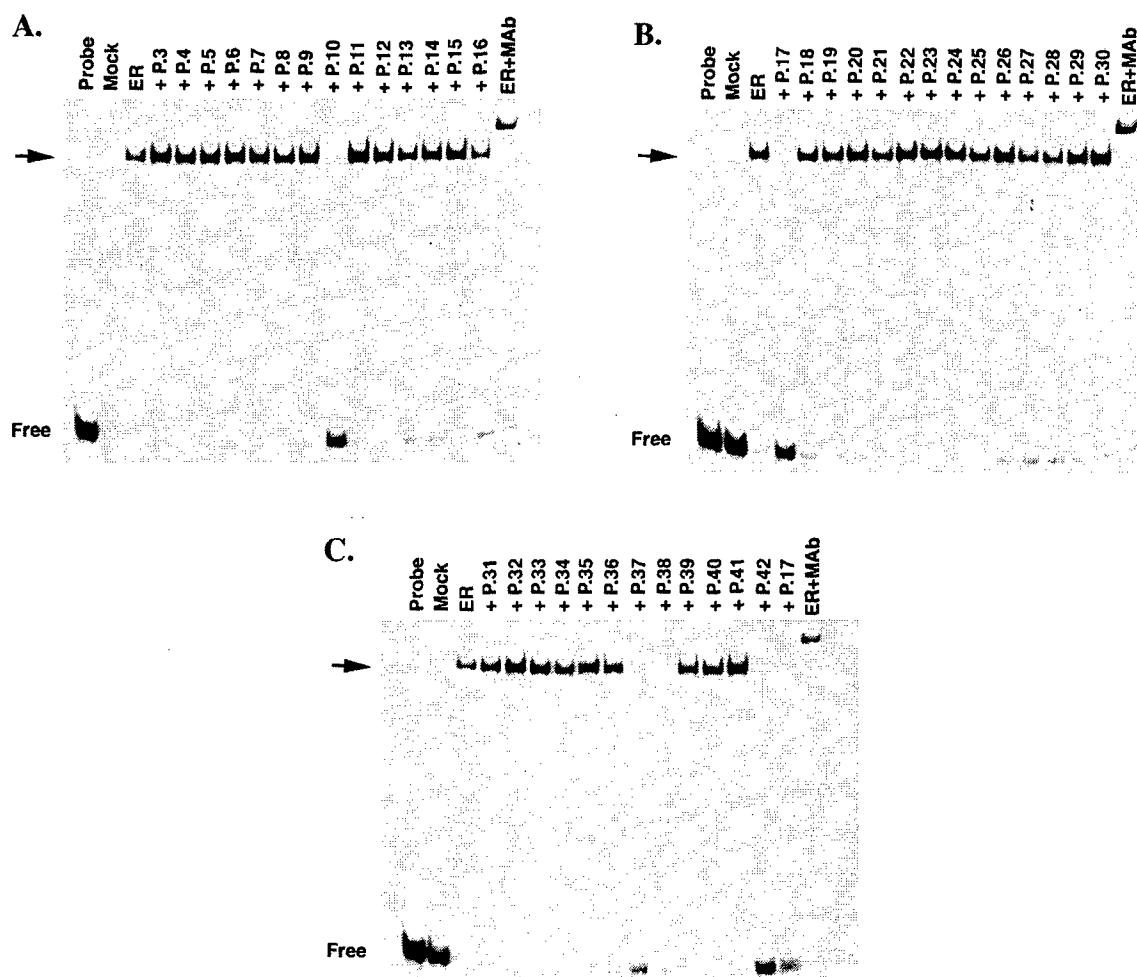


Fig. 3. Representative gel mobility shift experiments showing the effect of synthetic ER peptides on the DNA-binding activity of ER

In the absence of added peptide, ER expressed in Cos-7 cells binds efficiently and with high specificity to an ERE-containing oligonucleotide (lane 3 of each gel) compared with probe alone (lane 1 of each gel). Dependence of this complex on ER was confirmed by the absence of this complex when an equivalent amount of a mock-transfected extract from Cos-7 cells was used (lane 2 of each gel) and by the ability of an ER-specific monoclonal antibody to supershift this complex (final lane of each gel). Data summarizing the behavior of all 40 synthetic ER peptides is summarized in the bar graph shown in figure 4.

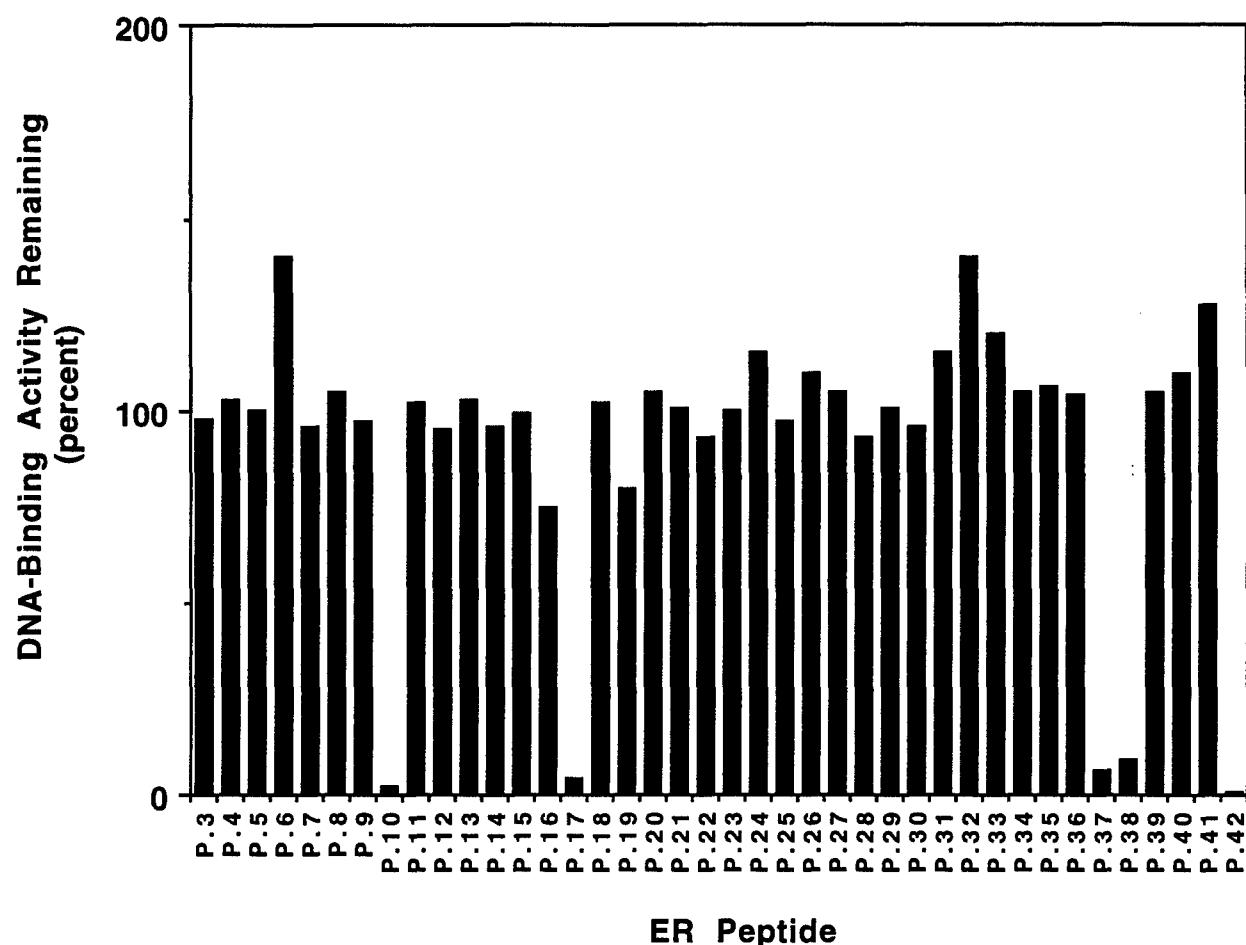


Fig. 4. DNA-Binding activities remaining after treatment of Cos-7 / ER extracts with 20 pmol of the indicated synthetic ER peptides

Our results indicate that the DNA-binding of activity ER can be dramatically inhibited by the presence of five discrete synthetic ER peptides. The ability of only a few of the 40 peptides to interfere with DNA-binding by ER strongly argues that this is a sequence-specific effect, rather than a result of non-specific inhibition of DNA-binding by high concentrations of peptide or by components of the buffer in which the peptides were dissolved. Sequences of the active peptides, which included P₁₀, P₁₇, P₃₇, P₃₈, and P₄₂, are summarized in Table I, below.

Table I. Amino Acid Sequence of Inhibitory ER Peptides

<u>Peptide Designation</u>	<u>Location within hER</u>	<u>AA sequence</u>
P-10	residues 507-518	Ac-LLLILSHIRHMS-NH ₂
P-17	residues 371-384	Ac-TLHDQVHLLECAWL-NH ₂
P-37	residues 1-20	NH ₂ -MTMTLHTKASGMALLHQIQC-NH ₂
P-38	residues 58-77	Ac-AAYEFNAAAAANAQVYQQTG-NH ₂
P-42	residues 497-516	Ac-LQQHQQLAQLLILSHIRH-NH ₂

In order to assess the relative inhibitory potency of these peptides, titration experiments were performed with decreasing concentrations of each of the five active peptides shown in Table I, along with representative inactive peptides. Data from typical titration experiments are presented in figure 5.

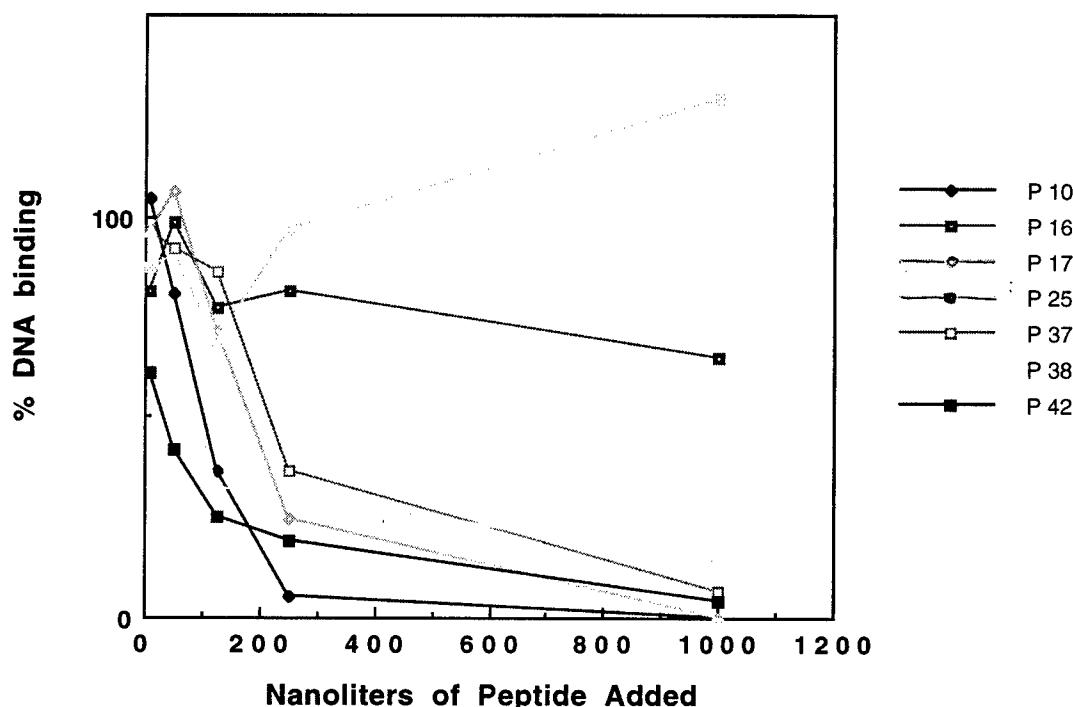


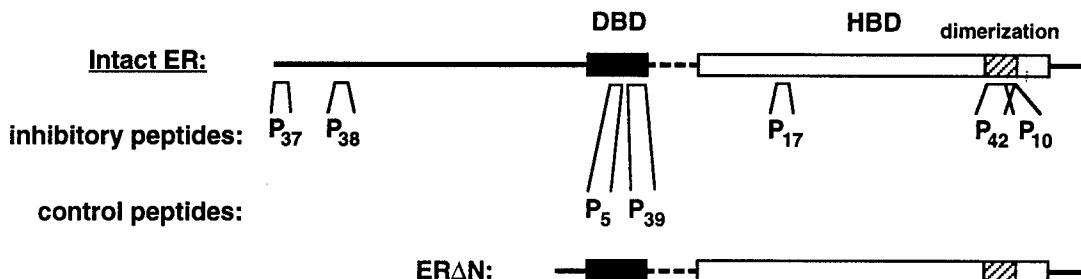
Fig. 5. Effect of increasing peptide concentrations on hER DNA-binding activity

It is noteworthy that the two peptides showing the greatest degree of inhibitory activity (P₁₀ and P₄₂) include overlapping sequences that contribute to the putative dimerization motif described by Parker and colleagues (7) in the murine ER. A third peptide (P₁₇) comes from further upstream in the ligand binding domain. The remaining two active peptides somewhat unexpectedly are derived from the amino terminal portion of hER, representing a region that has not previously been implicated in dimerization of receptor subunits or in coactivator interactions. The remaining 35 peptides had little if any reproducible effect on DNA-binding by ER in repeated experiments (see figures 3-5), indicating that the effect of the five inhibitory peptides is specific. Since each gel shift reaction utilized equivalent aliquots from a single ER-containing Cos-7 cell extract, the intensity of the bands corresponding to the ER/DNA complex is assumed to accurately reflect the amount of ER protein that remains available for binding to DNA after complexation with peptide.

Identifying the site of interaction within ER of the inhibitory peptides.

The ability of synthetic ER peptides to interfere with the DNA binding activity of intact ER in whole cell extracts suggests that they are able to physically interact with one or more regions of the ER polypeptide. To attempt to address this question and localize potential sites of interaction, the activity of selected inhibitory peptides was determined on an amino-terminally truncated ER mutant, ER Δ N (containing amino acids 174-595) versus intact ER (with amino acids 1-595). Like the full length receptor, ER Δ N is able to bind efficiently to DNA in a gel mobility shift assay (figure 6b) and can induce the expression of the pERE-TK-CAT reporter gene in a transient co-transfection assay (data not shown). Figure 6a compares the functional organization of ER Δ N with intact ER and shows the location of peptides used for this experiment. As seen before with the intact ER, P₁₀, P₁₇, and P₄₂ showed the greatest degree of inhibition of DNA-binding by ER Δ N compared to the buffer control, while P₃₇ and P₃₈ showed a significant, but less pronounced inhibitory effect (figure 6b). Peptides P₅ and P₃₉ were included as negative controls and were again without effect on ER Δ N. The ability of P₃₇ and P₃₈ to inhibit the DNA-binding activity of ER Δ N raises the interesting possibility that in its native state, ER is folded in such a way that its amino terminal region (containing P₃₇ and P₃₈) makes physical contact with sequences downstream of residue 174 within ER. Alternatively, these synthetic ER peptides may be interacting with proteins associated with ER (or with ER Δ N) that are necessary for its DNA binding activity, rather than with ER itself.

A.



B.

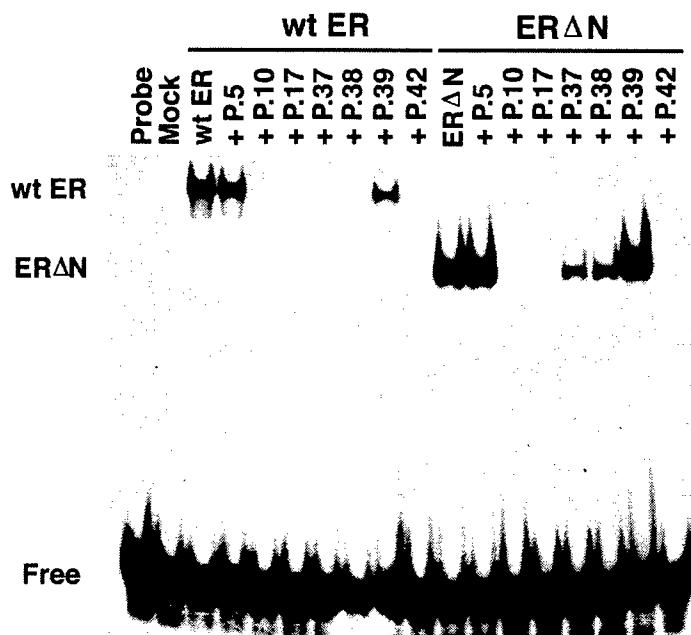


Fig. 6. Comparison of the inhibitory activity of synthetic ER peptides on DNA-binding by ER Δ N compared with wt ER

DNA-binding activity of amino acid substitution mutants of ER.

As a complementary approach to using synthetic peptides to identify "functionally critical surfaces" on the ER protein, we have analyzed the DNA-binding activity of a limited number of ER mutants containing amino acid substitutions within the hormone-binding domain. Sodium bisulfite mutagenesis was targeted using a gapped-duplex approach to enable the isolation of 11 point mutants within the ER sequences corresponding to peptides P_{10} , P_{17} , and P_{42} . These substitution mutants were then transferred to the pCMV4 expression vector to enable their transfection into Cos-7 cells for an assessment of their DNA-binding activity. Results from a typical gel mobility shift assay are given in figure 7. While not all of these amino acid substitution mutants appeared to disrupt the DNA-binding activity of ER, it is significant that a number of them did. Mutants that interfered with DNA binding included A505V and L511F. Of the remaining point mutants L378F and A382V showed slightly reduced DNA-binding activity, while T371I, L372F, H373Y, C381S, L509F, S512L, and H516Y were essentially indistinguishable from wild type ER.

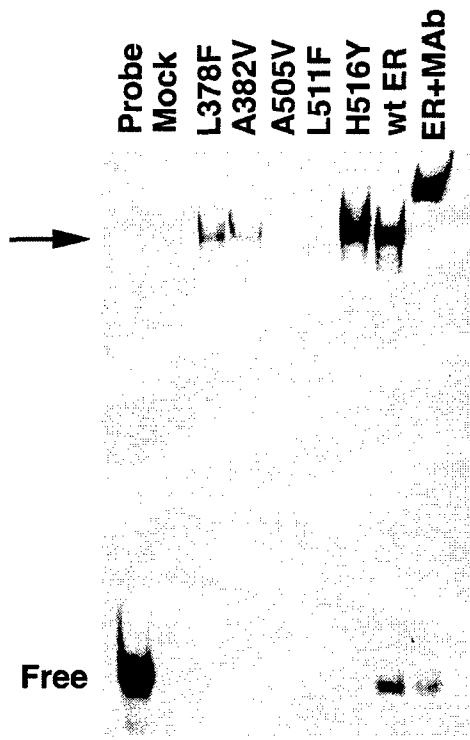


Fig. 7. Gel mobility shift assay showing measuring the DNA-binding activities of selected amino acid substitution mutants of ER

Introduction of synthetic ER peptides into transfected cells by electroporation.

Experiments to test the inhibitory potential of synthetic hER peptides when introduced into cultured cells were essentially unsuccessful. These experiments concentrated on the use of electroporation, since this represents a relatively non-specific biophysical method for introducing transient pores into the plasma membrane that should theoretically enable cellular uptake of small biomolecules such as plasmids and peptides. The success of this assay depends on successful and

efficient co-transfer of the synthetic peptides to be tested along with two plasmids (pCMV-ER and pERE-TK-CAT) that were necessary to measure an ER-dependent transcriptional response. A variety of electroporation conditions were examined, including a wide range of voltage and capacitance settings. Peptide P₄₂ was chosen for use in these pilot experiments, based on its strong inhibitory activity in the gel mobility shift assays. While we were able to routinely measure an E₂-dependent increase in CAT gene expression following electroporation of HeLa cells, we never observed significant inhibition of this response with added synthetic peptide. A number of experiments were also unsuccessfully attempted using Lipofectin (Gibco BRL, Bethesda, MD), a cationic lipid that is reported to be useful for transferring peptides as well as nucleic acids into cells.

Success with this specific aim (Task 2.d.) was substantially hindered by the inability to directly measure the efficiency of peptide uptake by cells. Since chemical modification of the peptides would run the risk of destroying their activity, this problem could only be solved by the synthesis and use of isotopically labeled peptides. Experiments of this nature were outside the budgetary and time constraints of this project and were therefore not attempted.

DISCUSSION

Five promising candidates for peptide inhibitors of the estrogen receptor were identified using an electrophoretic gel mobility shift assay to screen through a library of 40 synthetic peptides derived from the ER polypeptide sequence. Their location within the ER protein is summarized in figure 8. Inhibition of DNA binding by the peptides was observed to be specific and reproducible. This inhibition was concentration dependent, and appeared to be targeted largely to the hormone-binding domain of ER. This is consistent with a model that peptide binding to the dimerization interface within the ER hormone-binding domain can effectively block efficient DNA binding by interfering with subunit association. We have not attempted to directly measure the effect of synthetic peptides on dimerization of ER subunits in solution, although this is currently feasible using glutathione-S-transferase "pull-down" or antibody co-immunoprecipitation assays.

While a significant molar excess of peptide over receptor appears to be required for inhibition to be observed (see figure 4), the effects were seen over a range of peptide concentrations (estimated to be 0.02 to 2 microMolar) that represents a reasonable starting point for the development of a peptide-based pharmaceutical to serve as a non-hormonal disrupter of estrogen receptor function. Experiments to test the inhibitory potential of these peptides in a cell culture system for transcriptional regulation by ER were attempted, but were unsuccessful.

It is significant that mutation of several amino acid residues within the peptide sequences of P₁₇, and P₄₂ produce an impaired DNA-binding phenotype when they are present within the context of the full length ER. This observation further argues that these peptides are likely to correspond regions of exposed secondary and tertiary structure on the surface of the ER hormone-binding domain. Their behavior also argues that these peptides are able to assume a defined secondary structure in solution that resembles their structure in the intact ER protein. Notably, Bauman and Hard have used NMR and CD spectroscopy to show that a synthetic peptide corresponding to residues Leu⁴⁹⁷ to Asn⁵¹⁹ of human ER (roughly equivalent to P₄₂ used in this study) is able to form a structured alpha-helix in aqueous solution with a weak propensity to dimerize with itself (8). In contrast, a control peptide corresponding to human ER sequences Lys⁵²⁰ to His⁵²⁴ (contained within our inactive peptide P₁₄) had no propensity to form an α -helical conformation (8).

An X-Ray crystal structure for the hormone-binding domain of artificially expressed human ER α has recently been reported (9). Interestingly, this structure confirms that ER residues Leu⁴⁹⁷ to Asn⁵¹⁹ (corresponding to our overlapping peptides P₁₀ and P₄₂) make up a well defined α -helix (referred to as H-11) that lies on the surface of the hormone-binding domain. Residues mapping to H-11 were previously shown by Parker and colleagues to play an important role in subunit dimerization in mouse ER (7). It is worth noting that the A505V and L511F mutations that we observed to strongly disrupt the DNA-binding activity of ER also fall within this region, and presumably lie on the solvent-exposed dimer surface. Similarly, P₁₇ corresponds largely to α -helices 4 and 5 (H-4 and H-5) in the crystal structure of ER α which are disposed close to H-11 on the surface of the hormone-binding domain and contribute to the dimerization interface. Thus, there is excellent agreement between the known 3-dimensional structure of the hormone-binding domain of ER (9) and our data and that of Parker and colleagues (7) on mutation-sensitive sites within ER that disrupt DNA-binding. Furthermore these structures correspond well to peptides P₁₀, P₁₇, and P₄₂ that were observed in our hands to block DNA-binding activity when added to extracts containing ER. Unfortunately, structural data corresponding to the amino-terminal region of ER that contain peptides P₃₇ and P₃₈ is not yet available to assist in the interpretation of our findings.

A manuscript detailing the observations included in this report is in preparation. Based on our preliminary results, an invention discloser statement was filed with the Office of Intellectual Property of Michigan State University, as well as being forwarded to the US Army MRMC and the Office of Research Services of SUNY at Stony Brook. A preliminary institutional review of this disclosure statement and a search for the patentability of this invention was undertaken. The initial assessment was that this discovery would not be patentable in its present form, but this question remains under consideration. Copies of correspondences pertaining to this invention disclosure are included as Appendix B.

SUMMARY

Despite predictable delays and disruption caused during 1995 & 1996 by relocation of this project from SUNY at Stony Brook to Michigan State University, we can nonetheless report significant progress on this project. Our goal was to identify short peptides, derived from the sequence of the ER protein, that are able to interfere with the DNA-binding activity of this receptor. Five synthetic peptides were identified that share this inhibitory activity. The behavior of three of these peptides shows excellent agreement with recently reported structural information on ER and its dimerization surface. The underlying justification for this work continues to be the development of a novel class of steroid receptor inhibitors that may someday prove to be useful as therapeutic agents. If additional resources can be identified in the future, derivatives of these peptides should be made and tested for their inhibitory activity. New peptides should incorporate chemical modifications designed to increase cell permeability and *in vivo* stability. In addition, further work to improve methods for delivery of peptide-based drugs or peptide-mimetics to the cell interior would appear to be warranted. Our experiments demonstrate significant potential for the use of peptides to interfere with ER function. While our experiments have been limited to ER α , this strategy has general applicability for other members of the nuclear receptor family as well as for other classes of transcription factors.

Key Personnel Contributing to this Project

<u>Investigator</u>	<u>Role</u>	<u>Effort</u>	<u>Period</u>
Richard J. Miksicek, Ph.D.	Co-PI Responsible for Conduct of Research	25 %	9/94-10/97
Sandra Haslam, Ph.D.	Acting PI for MSU Subcontract, Consultant	5 %	7/95-10/97
Connie Lee	Technical Research Assistant (SUNY @ SB)	100 %	9/94-6/95
Mary Morrison	Senior Research Associate (MSU)	100 %	7/95-10/97

Publications During Current Award Year:

Morrison, M., Lee, C., Topp, K. and Miksicek, R.J. Inhibition of the DNA-binding activity of estrogen receptor- α by synthetic peptides designed to mimic receptor dimerization surfaces, manuscript in preparation.

Invention Disclosure:

MSU Invention Disclosure #96-066 "Peptide-Based Inhibitors of Estrogen Receptor Function",
Filed 08/26/96

REFERENCES

- 1) Miksicek, R.J. (1994) Steroid receptor variants and their potential role in cancer. *Seminars in Cancer Biology* **5**:369-379.
- 2) Andersson, S., Davis, D.N., Dahlback, H., Jornvall, H., and Russell, D.W. (1989) Cloning, structure, and expression of the mitochondrial cytochrome P450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* **264**:8222-8229.
- 3) Neff, S., Sadowski, C., and Miksicek, R.J. (1993) Site-directed mutagenesis of the hormone-binding domain of the human estrogen receptor reveals that two cysteine residues play a role in DNA-binding and subcellular localization. *Mol. Endocrinol.* **8**:1215-1223.
- 4) Shortle, D. and Bostein, D. (1983) *Methods in Enzymology* **100**:457-462.
- 5) Wang, Y. and Miksicek, R.J. (1991) Identification of a dominant negative form of the human estrogen receptor. *Mol. Endocrinol.* **5**:1707-1715.
- 6) Ince, B.A., Zhuang, Y., Wrenn, C.K., Shapiro, D.J., and Katzenellenbogen, B.S. (1993) Powerful dominant negative mutants of the human estrogen receptor. *J. Biol. Chem.* **268**:14026-14032.
- 7) Lees, J.A., Fawell, S.E., White, R., Parker, M.G. (1990) A 22-amino acid peptide restores DNA-binding activity to dimerization defective mutants of the estrogen receptor. *Mol. Cell. Biol.* **10**:5529-663.
- 8) Baumann, H. And Hard, T. (1995) Structural characterization of a short peptide fragment that mediates estrogen-receptor dimerization. *Eur. J. Biochem.* **230**:879-885.
- 9) Brzozowski, A.M., Pike, A.C.W., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.-A., and Carlquist, M. (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389**:753-758.

Appendix A: Sequences of Synthetic Peptides Obtained for this Project

* note, these peptides are numbered 3-42 based on increasing size (peptides 1 and 2 were synthesis controls), but they have been ordered by their position within the protein sequence or ER, beginning at the N-terminus.

<u>Number</u>		<u>Peptide Sequence</u>		<u>Size (aa residues)</u>
37)	free NH ₂ -	MTMLHTKASGMALLHQIQC	-amide	20
15)	acetyl-	GNELEPLNRPQLKI	-amide	14
7)	acetyl-	PLERPLGEVYL	-amide	11
8)	acetyl-	PLGEVYLDSSK	-amide	11
4)	acetyl-	PAVYNPEG	-amide	9
38)	acetyl-	AAYEFNAAAANAQVYQQTG	-amide	20
32)	acetyl-	LPYGPGSEAAFGSNGLG	-amide	18
33)	acetyl-	GFPPLNSVSPSPLMLLHP	-amide	18
9)	acetyl-	PPQLSPFLQPHG	-amide	12
11)	acetyl-	QQVPYYLENEPSG	-amide	13
12)	acetyl-	YTVREAGPPAFYR	-amide	13
29)	acetyl-	PNSDNRRQGGRRERLASC	-amide	17
23)	acetyl-	NDKGSMAMESAKETRY	-amide	16
16)	acetyl-	CNDYASGYHYGVWS	-amide	14
24)	acetyl-	CKAFFKRSIQGHNDYM	-amide	16
5)	acetyl-	CTIDKNRRKS	-amide	10
39)	acetyl-	CYEVGMMKGGIRKDRRGGRM	-amide	20
40)	acetyl-	LKHKRQRDDGEGRGEVGSAG	-amide	20
41)	acetyl-	DMRAANLWPSPLMIKRSKKN	-amide	20
36)	acetyl-	SLALSLTADQMVSAALLDAE	-amide	19
13)	acetyl-	PPILYSEYDPTRP	-amide	13
34)	acetyl-	FSEASMMGLLTNLADREL	-amide	18
25)	acetyl-	VHMINWAKRPGFVDL	-amide	16
17)	acetyl-	TLHDQVHLLECAWL	-amide	14
18)	acetyl-	EILMIGLVWRSMEH	-amide	14
26)	acetyl-	PGKLLFAPNLLDRNQ	-amide	16
30)	acetyl-	GKCVEGMVEIFDMLLAT	-amide	17
27)	acetyl-	SSRFRMMNLQGEEFVC	-amide	16
28)	acetyl-	LKSIILLNSGVYTFLS	-amide	16
35)	acetyl-	STLKSLEEKDHIHRVLDK	-amide	18
22)	acetyl-	ITDTLIHLMAKAGLT	-amide	15
42)	acetyl-	LQQQHQRLAQQLLILSHIRH	-amide	20
6)	acetyl-	LQQQHQRLAQ	-amide	10
10)	acetyl-	LLLILSHIRHMS	-amide	12
14)	acetyl-	MSNKGMEHLYSMK	-amide	13
3)	acetyl-	CKNVVPLY	-amide	8
19)	acetyl-	DLLLEMLDAHRLHA	-amide	14
20)	acetyl-	PTSRGGASVEETDQ	-amide	20
21)	acetyl-	SHLATAGSTSSHSL	-amide	20
31)	acetyl-	CQKYYITGEAEGFPATV	-free acid	17

**MICHIGAN STATE
UNIVERSITY**

DAMD 1794J441

MIKSICEK, Richard J.

Appendix B: Invention Disclosure Statement and Related Correspondence

August 29, 1996

MEMORANDUM

TO: RICHARD MIKSICEK *red*
FROM: FRED ERBISCH
SUBJECT: PEPTIDE INHIBITORS OF THE ESTROGEN RECEPTOR

Thank you for submitting the above listed invention disclosure. It has a receipt date in my office of August 26, 1996 and has been assigned **Invention Disclosure Number 96-066.**

FHE/dmb

cc: Dr. William J. Spielman



OFFICE OF
RESEARCH
AND
GRADUATE STUDIES

Office of Intellectual
Property

Michigan State University
238 Administration Building
East Lansing, Michigan
48824-1046

517/355-2186
FAX: 517/432-1171

**MICHIGAN STATE
UNIVERSITY**

**Richard J. Miksicek, Ph.D.
Asst. Professor of Physiology
108 Giltner Hall**

Phone: (517)-355-6475 ext 1285
FAX: (517)-355-5125
email: miksicek@pslvax.psl.msu.edu

Memorandum

To: Jan Sherman
Office of Intellectual Property
238 Administration Building
Michigan State University
From: Richard Miksicek
Subject: Invention Disclosure
Date: August 23, 1996



DEPARTMENT OF
PHYSIOLOGY
Michigan State University
108 Giltner Hall
East Lansing, Michigan
48824-1101
517/355-6475
FAX: 517/355-5125

Dear Ms. Sherman,

Attached, please find a completed invention disclosure form for early phase development of a novel class of estrogen inhibitors that I'd like to submit for review by the Office of Intellectual Property. As we learn more about the activity and limitations of this inhibitor, there is a strong possibility that we would like to pursue an application to patent these compounds and the concept behind their development. In addition, any assistance that I could receive from your office in identifying biotechnology or pharmaceutical firms that might be interested in either a) funding further development of this project or b) collaborating with respect to the synthetic chemistry involved in further development would be greatly appreciated.

Thank you for your assistance.



MICHIGAN STATE UNIVERSITY INVENTION DISCLOSURE FORM

ID Number _____

Date of Receipt _____

1. Title of Invention: Peptide Inhibitors of the Estrogen Receptor

2. Name(s) of Inventor(s): Richard J. Miksicek, Ph.D. Physiology, CMM
Name _____ Unit _____
Name _____ Unit _____
Name _____ Unit _____
Name _____ Unit _____

3. Sponsor (check all that apply):

yes X no _____ University funds, facilities or equipment used
yes X no _____ non-University sponsor
sponsoring agency US Army, Dept. of Defense
University research account number 61-5507
yes _____ No X other, describe _____

4. Disclosure:

yes _____ no X Invention has been disclosed to non-
University personnel; if yes,
To whom: _____
Form of disclosure: oral presentation _____
written format _____
yes _____ no X Was there a confidentiality agreement
existing at time of disclosure?
Date disclosed: _____
yes X no _____ Disclosure, publication or presentation,
planned; if yes,
When: late in 1996
Where: Proc. Natl. Acad. Sci, (ms in preparation)

5. Date of Conception of the invention: July 1993

6. Have any prototypes, samples, etc., been made? If so, when?
Prototype synthetic peptides synthesized during 1995 are under continuing
investigation.

7. Use additional sheets to respond to the following and attach these sheets to this Cover Sheet.

- A. Describe in general terms the objectives of the invention.
- B. List the advantages of this invention over previous approaches. What is the "old" method? What specific features of this invention make it more advantageous than the previous approaches? What markets could use this invention?
- C. Describe in detail the steps that need to be taken to implement or construct the invention (attach sketch if applicable). Are there alternate methods for implementation and/or construction?
- D. What is the stage of development of the invention now? Describe plans for continued future research and/or study on the invention.
- E. List any key words to describe the invention.
- F. Include any other information that is pertinent to the invention.

8. All inventors should sign below; if necessary attach an additional signature sheet.

	August 21, 1996		
Signature	Date	Signature	Date
Richard J. Miksicek, Ph.D.			
Typed or printed name	Typed or printed name		

Signature	Date	Signature	Date
Typed or printed name		Typed or printed name	

9. This Invention Disclosure should be reviewed and witnessed after preparation by one knowledgeable in the field of the invention. The witness should sign below:

Disclosed to and understood by me, _____

Signature	Date
Typed or printed name	

MICHIGAN STATE UNIVERSITY INVENTION DISCLOSURE FORM

Title: Peptide Inhibitors of the Estrogen Receptor
Inventor: Richard J. Miksicek, Ph.D.

7. Description of Invention:

A. Objectives: The estrogen receptor serves as the crucial biochemical target for regulation of gene expression by estrogenic steroids. Its biological activity depends directly on its ability to interact with itself (in the form of homologous receptor dimers) and with other gene regulatory proteins to form higher order complexes that are capable of binding to DNA and promoting changes in gene transcription. This disclosure statement describes the development of short, synthetic peptides derived from the predicted protein sequence of the human estrogen receptor that are able to antagonize the effects of estrogen by virtue of their ability to interact directly with the estrogen receptor, or its associated transcription factors, thereby preventing functional receptor complexes from forming. The originality of this invention is the development of peptide-based inhibitors of estrogen receptor function with a novel mechanism of action. A preliminary list of candidate peptides that reproducibly display inhibitory activity *in vivo* includes:

hER peptide 10: N-acetyl-leucyl-leucyl-isoleucyl-leucyl-seryl-histidyl-isoleucyl-arginyl-histidyl-methionyl-serine amide

hER peptide 17: N-acetyl-threonyl-leucyl-histidyl-aspartyl-glutaminyl-valyl-histidyl-leucyl-leucyl-glutamyl-cysteinyl-alanyl-tryptophanyl-leucine amide

hER peptide 37: methionyl-threonyl-methionyl-threonyl-leucyl-histidyl-threonyl-lysyl-alanyl-seryl-glycyl-methionyl-alanyl-leucyl-histidyl-glutaminyl-isoleucyl-glutamyl-cysteine amide

hER peptide 38: N-acetyl-alanyl-alanyl-tyrosyl-glutamyl-phenylalanyl-asparaginyl-alanyl-alanyl-alanyl-alanyl-alanyl-asparaginyl-alanyl-glutaminyl-valyl-glycyl-glutaminyl-threonyl-glycine amide

hER peptide 42: N-acetyl-leucyl-glutaminyl-glutaminyl-histidyl-glutaminyl-arginyl-leucyl-alanyl-glutaminyl-leucyl-leucyl-isoleucyl-leucyl-seryl-histidyl-isoleucyl-arginyl-histidine amide

B. Advantages over previous approaches: All heretofore developed estrogen receptor inhibitors (estrogen antagonists) act as competitive hormone antagonists that prevent estradiol and other naturally occurring estrogens from binding to and activating its receptor target. As such, known estrogen antagonists are either steroids or steroid-like polycyclic phenols with chemically similar behavior. This invention discloses the discovery of short polypeptides that appear to be capable of exerting the same inhibitory effect on estrogen receptor function as estrogen antagonists, but via a distinctly different mechanism of action that does not involve the blockade of hormone binding. Therefore, drugs developed to mimic the effects of these inhibitory peptides would be expected to display distinct pharmacokinetic properties and a distinct profile of resistance compared with conventional anti-estrogens. In addition, since several of these peptides appear to be targeted to domains of

the estrogen receptor that are not involved in ligand (estrogen) binding, it is likely that they may provide the basis for the development of drugs that are also useful for inhibiting structural receptor variants, splicing variants, and receptor isoforms against which conventional estrogen antagonists are ineffective. The most likely applications for this class of drugs would be the treatment of hormone-dependent cancers of the breast and reproductive tract, or the treatment of proliferative disorders such as leiomyomas or endometriosis.

C. Steps needed to implement invention:

- 1) synthesis of peptide prototypes for pilot experiments (completed)
- 2) screening of candidate peptides for those that display inhibitory activity using an *in vitro* assay (completed)
- 3) documenting the peptide- and receptor-specificity of this inhibitory activity (in progress)
- 4) documenting the *in vivo* activity of inhibitory peptides using a model cell culture assay system (in progress)
- 5) precise delineation of minimal sequences required for inhibitory activity and further delineation of structure / activity requirements (planned)
- 5) determination of the 3-dimensional structure of selected active inhibitory peptides (will depend on future collaboration)
- 6) *de novo* chemical synthesis of small mimetic drugs incorporating crucial structural features of selected inhibitory peptides (will depend of future collaboration)
- 7) *in vitro*, cell culture, and animal testing of the inhibitory activity of selected peptide mimetics (will depend on future collaboration)

D. Developmental stage: As noted above, five short synthetic peptides have been identified that display a reproducible ability to interfere with DNA-binding by the human estrogen receptor. Work is in progress to further characterize the mechanism of this inhibition and to delineate the minimal structural requirements necessary for activity. Movement into a more focused drug development phase (involving the analysis of peptide structure and the design and testing of peptide mimetics) will require the future development of a collaboration, preferably with an interested biotechnology or pharmaceutical firm.

E. Keywords: Breast Cancer, Estrogen Antagonists, Peptide Inhibitors, Peptide Mimetics, Estrogen Receptor, Nuclear Receptors, Dimerization

F. Other information: Development of this invention is currently being funded by a US Army Breast Cancer Research Grant (DAMD 1794-J-4411). An application for a follow-up project to continue this work will be submitted in September, 1996 to the National Institutes of Health.

IAN C. MCLEOD, P.C.
ATTORNEY AND COUNSELOR AT LAW
PATENTS, TRADEMARKS AND COPYRIGHTS

IAN C. MCLEOD

MARY M MOYNE

1996 September 10

2190 COMMONS PARKWAY
OKEMOS, MICHIGAN 48864

TELEPHONE (517) 347-4100
TELECOPIER (517) 347 4103

Janyth M. Sherman, Licensing Associate
Office of Intellectual Property
Michigan State University
238 Administration Building
East Lansing, Michigan 48824-1046

Re: Michigan State University 1.7-328
(ID96-066)
Title: Peptide Inhibitors of
the Estrogen Receptor
Inventor: Richard J. Miksicek

Dear Jan:

I have set up the above referenced file and will conduct a patentability search in the near future.

If there are any questions in the meantime, please let me know.

Best wishes.

Sincerely,



Ian C. McLeod
ICM/ejm

cc: Dr. Richard J. Miksicek



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

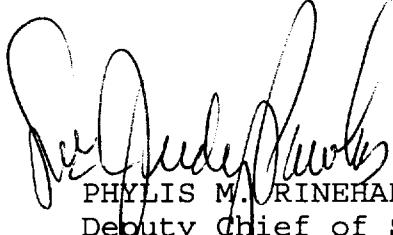
14 Oct 99

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grant DAMD17-94-J-4411. Request the limited distribution statement for Accession Document Numbers ADB240174 and ADB236438 be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.
2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by email at Judy.Pawlus@amedd.army.mil.

FOR THE COMMANDER:


PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

*Completed
2-8-2000
B.W.*